REMARKS

Claims 92 and 129-131 are pending and currently under examination. Claims 92, 130, and 131 were rejected under 35 U.S.C. § 112, first paragraph, as lacking adequate written description, claims 92 and 129-131 were rejected under 35 U.S.C. § 112, first paragraph, as lacking enablement over the full scope of the claims, and claims 92, 129, and 131 were rejected under 35 U.S.C. § 102(b) for anticipation over Tang et al. (WO 01/57190). Each of these rejections is addressed below.

Claim amendments

Claim 92 has been amended to cancel reference to the treatment of neuropathic pain. Claims 130 and 131 have been cancelled. No new matter is added by these amendment.

Rejections under 35 U.S.C. § 112, first paragraph, written description

Claims 92, 130, and 131 have been rejected as lacking adequate written description. The Office states "one skilled in the art cannot reasonably visualize or predict what critical amino acid residues would structurally characterize the genus of polypeptides required to be used in the claimed method. . . In otherwords, the specification fails to describe a single critical amino acid residue required for any definable function in the claimed genus." (Office action, page 4). In support of this rejection, the Office cites two Federal Circuit cases: Fiers v. Revel, 25 USPQ2d 1601, 1606 (Fed. Cir. 1993) and Univ. California v. Eli Lilly and Co., 43 USPQ2d 1398 (Fed. Cir. 1997). However, the facts of these two cases are distinguishable from the present case as the present specification discloses sequences of several NsG33 proteins and provides significant guidance as to which amino acids may be varied. Furthermore, the claims are limited to proteins that share at least 95% sequence identity with SEQ ID NO:4 and have certain conserved cysteine residues.

In both Fiers and Univ. of California, the claims at dispute were to a cDNA corresponding to a previously identified protein (human fibroblast beta-interferon and human insulin, respectively). In neither case did the specification disclose a single cDNA sequence that corresponded to the desired protein. In each case, the court ruled that while it may have been

within the skill of one in the art to obtain the claimed cDNA based on the disclosure of the specification, they failed to describe any structural features of the cDNA that would demonstrate possession of the invention.

In contrast to the claims at issue in Fiers and Univ. of California, which were directed to a cDNA sequence that was not disclosed at all in the specification, claim 92 is directed to the use of a genus of polypeptides that are structurally and functionally defined in the specification. Applicants here have disclosed the polypeptide sequence being claimed and have placed limits on the scope of that sequence. Namely, the polypeptide sequence recited in claim 92 is structurally restricted in two ways: the sequence must be 95% identical to the disclosed SEQ ID NO:4 and it must contain the recited conserved cysteine residues. Furthermore, the specification discloses a sequence alignment of NsG33 polypeptides derived from several species in figure 3 (mouse, rat, and human). The identification of amino acid residues conserved amongst the disclosed species in figure 3 provides guidance to the skilled artisan as to which structural features of the NsG33 are likely to be important to its function. Furthermore, the specification contains substantial guidance regarding amino acid substitutions which would be likely to preserve the desired activity of the claimed protein (see, e.g., page 21 of the specification). In view of this guidance provided by the specification, one skilled in the art at the time of filing would recognize that the Applicants had possession of the claimed invention over the full scope of the claims. The rejection of claim 92 for lack of written description should be withdrawn.

Rejections under 35 U.S.C. § 112, first paragraph, enablement

Claims 92 and 129-131 were rejected for lack of enablement over the full scope of the claims. Claims 130 and 131 have been cancelled, rendering the rejection of these claims moot. The Office states that the specification, "while being enabling for a method of treating a definable population of neurons with a structurally and functionally definable NsG33 polypeptide, does not reasonably provide enablement for treating unknown functions in unknown neuronal populations in patients with Huntington's disease or neuropathic pain using structurally and functionally undefined NsG33 polypeptides." (Office action, page 5.) The Office further states that Applicants' data is restricted to one population of neuronal cells in the putatmen, and

that "Huntington's disease is characterized by dysfunctional neurons in other areas of the brain." (Id.). Applicants respectfully traverse this rejection.

As noted above, the specification provides ample guidance to one skilled in the art to enable them to select functional polypeptides from the genus of claimed polypeptides having at least 95% sequence identity to NsG33 (i.e., SEQ ID NO:4) and the claimed conserved cysteine residues. Further, the specification provides methods for confirming the functionality of the claimed polypeptides (e.g., example 15), which enable one skilled in the art to separate functional from non-functional polypeptides with a minimum of experimentation. Therefore, the method of claim 92, which requires administration of a polypeptide having at least 95% identity to the sequence of SEQ ID NO: 4 and the recited conserved cysteine residues, is fully enabled by the specification.

The second aspect of the Office's rejection is based on the observation that Huntington's disease is a complex disease affecting several different types of neuronal cells. In order to satisfy the enablement requirement with respect to the claimed treatment, the Applicants are not required to show that every aspect of Huntington's disease is affected by the claimed method. Rather, the Applicants can satisfy the enablement requirement through testing in an *in vitro* model that reasonably correlates to the disease to be treated. See, e.g., M.P.E.P. § 2164.02, which states

> The issue of "correlation" is related to the issue of the presence or absence of working examples. "Correlation" as used herein refers to the relationship between *in vitro* or *in vivo* animal model assays and a disclosed or a claimed method of use. An *in vitro* or *in vivo* animal model example in the specification, in effect, constitutes a "working example" if that example "correlates" with a disclosed or claimed method invention. If there is no correlation, then the examples do not constitute "working examples." In this regard, the issue of "correlation" is also dependent on the state of the prior art. In other words, if the art is such that a particular model is recognized as correlating to a specific condition, then it should be accepted as correlating unless the examiner has evidence that the model does not correlate. Even with such evidence, the examiner must weigh the evidence for and against correlation and decide whether one skilled in the art would accept the model as reasonably correlating to the condition. *In re Brana*, 51 F.3d 1560, 1566, 34 USPQ2d 1436, 1441 (Fed. Cir. 1995) (reversing the PTO decision based on finding that *in vitro* data did not support *in vivo* applications).
>
> Since the initial burden is on the examiner to give reasons for the lack of enablement, the examiner must also give reasons for a conclusion of lack of correlation for an *in*

vitro or *in vivo* animal model example. A rigorous or an invariable exact correlation is not required, as stated in *Cross v. Iizuka*, 753 F.2d 1040, 1050, 224 USPQ 739, 747 (Fed. Cir. 1985):

> [B]ased upon the relevant evidence as a whole, there is a reasonable correlation between the disclosed *in vitro* utility and an *in vivo* activity, and therefore a rigorous correlation is not necessary where the disclosure of pharmacological activity is reasonable based upon the probative evidence. (Citations omitted.) (Underline added).

As evidence of enablement, Applicants' specification provides data showing the protection of striatal neurons *in vitro* using an art recognized assay (Example 15). From these data it is clear that the presence of NsG33 in a culture of striatal neurons significantly increases the percentage of b-III-tubulin positive neurons. This increased neuronal number may result from increased differentiation of neuronal progenitor cells present in the cultures and/or a survival effect on the differentiated neurons. These data are consistent with general neuroprotective and regenerative effect of NsG33.

One skilled in the art would have recognized that these results correlate with a candidate therapeutic being useful for treating Huntington's disease because those skilled in the art would have found that positive results obtained in the *in vitro* assay used by Applicants correlates with treatment efficacy. For example, Mizuno et al. (Dev. Biol. 165:243 (1994)) and Ventimiglia et al. (Eur. J. Neurosci. 7:213 (1995)) (abstracts submitted herewith) used a similar assay to test the effect of different neurotrophic factors (BDNF, NT-3 and NT-4/5) on the survival and differentiation of striatal neurons. Based on the results of Mizuno et al. and Ventimiglia et al., Anderson et al. (Proc. Natl. Acad. Sci 93:7346 (1996); submitted herewith) tested the effect of brain-derived neurotrophic factor (BDNF) in the Quinolinic Acid animal model of Huntington's Disease. The authors stated: "[r]ecent findings that several neurotrophic factors, including BDNF and NT-3, support the survival and morphological differentiation of GABAergic striatal neurons *in vitro* [Mizuno et al. and Ventimiglia et al.] prompted us to determine whether neurotrophic factors can protect striatal output neurons in an excitotoxic model of Huntington's disease." (Page 7346, end of first column).

Therefore, the findings of Mizuno et al. and Ventimiglia et al. provided a basis for Anderson et al. to test the effect of BDNF in a well established *in vivo* model of Huntington's

Disease (the Quinolinic Acid animal model). Hence, it was well known in the art that a positive effect on the survival and differentiation of striatal neurons *in vitro* correlates with a positive effect in treating Huntington's Disease.

As further evidence of enablement, Applicants have subsequently tested NsG33 in an animal model of Huntington's disease as described in the attached article (Jørgensen et al., "Lentiviral delivery of Meteorin protects striatal neurons against excitotoxicity and reverses motor deficits in the quinolinic acid rat model", Neurobiology of Disease, in press 2010; submitted herewith), which presents data showing that NsG33 also holds strong therapeutic potential *in vivo* for treatment of Huntington's disease.

While these data were obtained after the present filing date, it provides evidence of the sufficiency of the *in vitro* model disclosed in the specification for identifying candidate therapeutics for Huntington's disease. Accordingly, Applicants have utilized a strategy for evaluating the potential of a given compound as a drug candidate by:

- evaluating the effect of said compound *in vitro* on relevant cell cultures;
- selecting the compound with the desired effect; and
- evaluating the effect of the positive compound(s) in a relevant *in vivo* animal model.

These submissions demonstrate that the data disclosed in the present specification are sufficient to enable one skilled in the art to practice the claimed invention with a minimum of experimentation. The possibility, as set forth by the Office, that other cell types or therapeutic targets might exist for possible Huntington's disease therapy that are not contemplated by the specification is not material to the enablement of the claimed methods, as the evidence presented by Applicants clearly show that one of skill in the art would have accepted Applicants' data as reasonably correlating with treatment efficacy in Huntington's disease. Therefore, the rejection of claims 92 and 129 for lack of enablement should be withdrawn.

Finally, the Office separately argues that methods of treating neuropathic pain were not enabled by the specification. Solely to expedite prosecution, Applicants have amended claim 92 to remove treatment of neuropathic pain. Therefore, this rejection is rendered moot.

Rejections under 35 U.S.C. §102(b)

Claims 92, 129, and 131 have been rejected as being anticipated by Tang. Solely to expedite prosecution, claim 92 has been amended to incorporate the limitations of claim 130, and claim 131 has been cancelled. Consequently, the rejection for anticipation is moot and should be withdrawn.

CONCLUSION

Applicant submits that the claims are in condition for allowance and such action is respectfully requested.

If there are any charges or any credits, please apply them to Deposit Account No. 03-2095.

Respectfully submitted,

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Neurobiology

Ciliary neurotrophic factor protects striatal output neurons in an animal model of Huntington disease

(brain-derived neurotrophic factor/excitotoxicity/nerve growth factor/neurotrophin-3/quinolinic acid)

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ABSTRACT **Huntington disease is a dominantly inherited, untreatable neurological disorder featuring a progressive loss of striatal output neurons that results in dyskinesia, cognitive decline, and, ultimately, death. Neurotrophic factors have recently been shown to be protective in several animal models of neurodegenerative disease, raising the possibility that such substances might also sustain the survival of compromised striatal output neurons. We determined whether intracerebral administration of brain-derived neurotrophic factor, nerve growth factor, neurotrophin-3, or ciliary neurotrophic factor could protect striatal output neurons in a rodent model of Huntington disease. Whereas treatment with brain-derived neurotrophic factor, nerve growth factor, or neurotrophin-3 provided no protection of striatal output neurons from death induced by intrastriatal injection of quinolinic acid, an *N*-methyl-D-aspartate glutamate receptor agonist, treatment with ciliary neurotrophic factor afforded marked protection against this neurodegenerative insult.**

Glutamate receptor-mediated excitotoxicity has been hypothesized to play a role in numerous neurodegenerative diseases, including Huntington disease and motor neuron disease (1, 2). The predominant neuropathological feature of Huntington disease is a massive degeneration of the medium-sized, γ-aminobutyric acid (GABA)ergic, striatal output neurons, without substantial loss of striatal interneurons (3, 4). The preferential loss of striatal output neurons observed in Huntington disease, and the resulting dyskinesia, are mimicked in rodent or primate models in which an *N*-methyl-D-aspartate (NMDA) glutamate receptor agonist, quinolinic acid, is injected into the striatum (1).

The search for therapeutic neuroprotective agents has led to the discovery that several classes of endogenous proteins that promote the survival and differentiation of embryonic neurons also possess neuroprotective and restorative properties when administered to adult animals (5–11). This has been demonstrated most comprehensively for motor neurons, where neurotrophic factors such as brain-derived neurotrophic factor (BDNF), neurotrophin-3 (NT-3), ciliary neurotrophic factor (CNTF), or glial-derived neurotrophic factor have been shown to support survival of these cells *in vitro*, and to preserve them *in vivo* following axotomy or in mutant mouse strains exhibiting spontaneous motor neuron degeneration (12–19). Recent findings that several neurotrophic factors, including BDNF and NT-3, support the survival and morphological differentiation of GABAergic striatal neurons *in vitro* (20, 21) prompted us to determine whether neurotrophic factors can protect striatal output neurons in an excitotoxic model of Huntington disease.

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MATERIALS AND METHODS

Trophic Factors. Recombinant human BDNF, nerve growth factor (NGF) and NT-3, and recombinant rat CNTF were prepared in *Escherichia coli* and characterized as described (22, 23). Axokine1 (Ax1) is the designation for recombinant human CNTF with the following modifications: substitutions of alanine for cysteine at position 17 and arginine for glutamine at position 63, and deletion of the 13 C-terminal amino acids. The 63QR substitution confers a 4–5-fold greater potency *in vitro* relative to human CNTF (24), and the other modifications confer stability for at least a week at 37°C in physiological buffer (data not shown).

Animal Treatments. All animal procedures were conducted in strict compliance with protocols approved by the institutional animal care and use committee.

Trophic factor delivery by osmotic pump. A 30-gauge osmotic pump infusion cannula and a 22-gauge guide cannula (5.0 and 2.2 mm long, respectively) were chronically implanted side-by-side into the left hemisphere (stereotaxic coordinates AP 0.7, ML 3.2 relative to bregma; incisor bar 3.3 mm below the interaural line) in 250–300 g male, Sprague–Dawley rats under deep chloral hydrate (170 mg/kg) and pentobarbital (35 mg/kg) anesthesia. Thirty days later, the rats were again anesthetized and an Alzet osmotic minipump 2002 (2-week capacity at a delivery rate of 0.5 μl/hr), containing 0.1 M PBS (pH 7.4), or PBS solutions of recombinant human NGF (0.9 mg/ml), human BDNF (1 mg/ml), human NT-3 (1 mg/ml), rat CNTF (0.78 mg/ml), or Ax1 (0.4 mg/ml) was connected by plastic tubing to the infusion cannula and implanted subcutaneously (25). Due to the dead volume of the infusion cannula and tubing, the delivery of neurotrophic factor into the brain began about 1 day after pump implantation. Neurotrophins maintained in osmotic pumps at 37°C for 12 days were completely stable, as determined by bioassay, and effective intrastriatal delivery of the neurotrophins was verified by immunohistochemical staining of sections for the appropriate factor (25). Three or four days after pump implantation, anesthetized rats received an injection of quinolinic acid [50 nmol in 1 μl phosphate buffer (pH 7.2), over 10 min] through the guide cannula using a 10-μl Hamilton syringe with a 28-gauge blunt-tipped needle.

Trophic factor delivery by daily injection. A 22-gauge guide cannula (2.2 mm long) was chronically implanted into the left hemisphere (stereotaxic coordinates AP 0.5, ML 3.0) of anesthetized rats, as described above. Beginning 1 week later, anesthetized rats received a daily intrastriatal injection of Ax1 (0.4 μg in 1 μl, over 10 min) or vehicle through the guide

Abbreviations: Ax1, Axokine1; BDNF, brain-derived neurotrophic factor; CNTF, ciliary neurotrophic factor; GABA, γ-aminobutyric acid; NMDA, *N*-methyl-D-aspartate; NGF, nerve growth factor; NT-3, neurotrophin-3.

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cannula using a Hamilton syringe. Ax1 was injected for 3 consecutive days before and 1 day after injection of quinolinic acid, which was injected as described above.

Histological Procedures and Analysis. Brains perfusion-fixed in 4% paraformaldehyde were collected 8 or 9 days after the quinolinic acid injection, and cut in the coronal plane into 40-micron thick sections that were stained with thionin. In each experiment, a series of 1 in 12 Nissl-stained sections was evaluated by an investigator unaware of treatment conditions, and the relative loss of medium-sized striatal neurons was rated on the following scale: 0 (no neuron loss), 1 (clear but slight neuron loss), 2 (moderate neuron loss), 3 (severe but not total neuron loss), 4 (total loss of medium-sized neurons within the field of the quinolinic acid injection). In cases where neuron loss appeared intermediate to two criteria, a half score between the two closest scores was assigned. Neuron loss scores that were assigned independently by two different observers in the experiments using BDNF and NT-3 were within 0–0.5 point of each other for 40 of 42 rats (correlation coefficient = 0.8; $P = 0.0001$).

In the experiment using CNTF, neuron loss also was evaluated by counting neurons in sections taken 0.5 mm rostral to the infusion cannula. For each section, neurons were counted that intersected every vertical line of a 10 × 10 sampling grid placed over seven fields, 0.4 × 0.4 mm, within the treated striatum. The first field was located slightly lateral to the center of the striatum, at the center of a typical quinolinic acid-induced lesion (i.e., immediately rostral to the tip of the infusion cannula). The six other fields were selected by moving diagonally from the first field, twice each in the dorsomedial and the ventromedial directions, and once each in the dorsolateral and the ventrolateral directions. To control for possible variation in section thickness, seven fields in equivalent locations were sampled in the contralateral striatum (≈600 neurons counted per 7 fields), and neuron survival was expressed as a percentage of neurons on the treated side relative to the intact side. The results of actual neuron counts (31 and 71% neuron loss for CNTF- and PBS-treated groups, respectively) showed close agreement with the results of the neuron loss scoring system (mean neuron loss scores of 1.67 and 3.25, respectively), as assessed by regression analysis (Spearman rank correlation coefficient = 0.82, $P < 0.05$).

Differences between experimental groups and their respective control groups were evaluated by unpaired t test.

RESULTS

In a series of experiments, quinolinic acid (50 nmol) was injected into the left striatum of adult rats 3 or 4 days after the start of intrastriatal infusion of neurotrophic factor by osmotic pump (nominal delivery rates: human NGF, 10.8 μg/day; human BDNF or NT-3, 12.0 μg/day; rat CNTF, 9.4 μg/day). This dose of quinolinic acid is toxic to medium-sized striatal output neurons, which constitute over 90% of all striatal neurons, yet leaves the striatal populations of cholinergic interneurons and parvalbumin/GABAergic interneurons largely intact (26, 27). Microscope analysis of Nissl-stained sections from brains collected 8–9 days after injection of quinolinic acid showed no significant sparing of medium-sized striatal neurons in BDNF-, NGF-, or NT-3-treated brains (Fig. 1). In an additional set of experiments, no neuron sparing was apparent when quinolinic acid was injected 7 days after the start of BDNF or NGF infusion (data not shown).

In striking contrast, neuron survival was significantly greater in rats treated with CNTF compared with rats treated with vehicle alone (Fig. 2), as determined by neuron counts that demonstrated a mean percent survival (±SEM) of 69 ± 17 and 29 ± 11%, respectively [unpaired t test, $t(5) = 2.12$, $P = 0.04$],

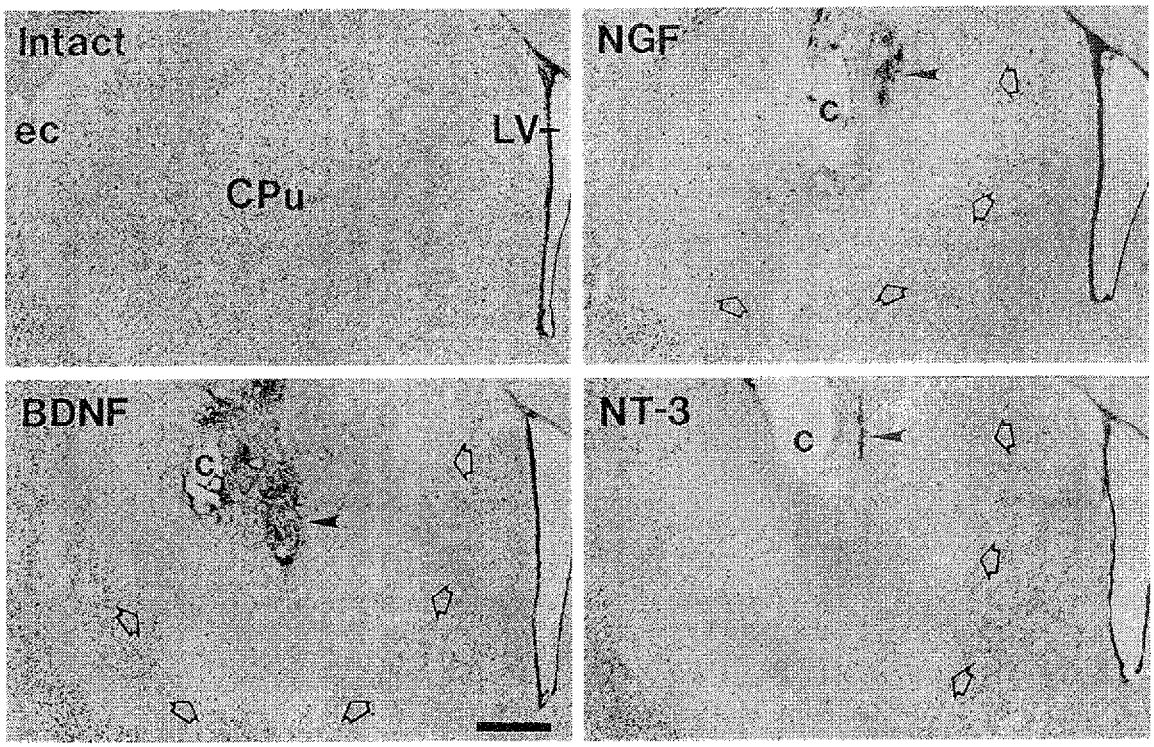

FIG. 1. Representative Nissl-stained sections (coronal plane) from brains treated with neurotrophins and injected with quinolinic acid. (*Upper left*) View of an intact caudate putamen (CPu). Adjacent panels: comparable views of sections from brains treated with NGF, BDNF, or NT-3 and injected with quinolinic acid. In the neurotrophin-treated brains, a circumscribed area (indicated by open arrows) is virtually devoid of medium-sized neurons. The two tracks in the caudate putamen were left by the infusion cannula (c) and the quinolinic acid injection needle (arrowhead). ec, external capsule; LV, lateral ventricle. (Scale bar = 0.5 mm.)

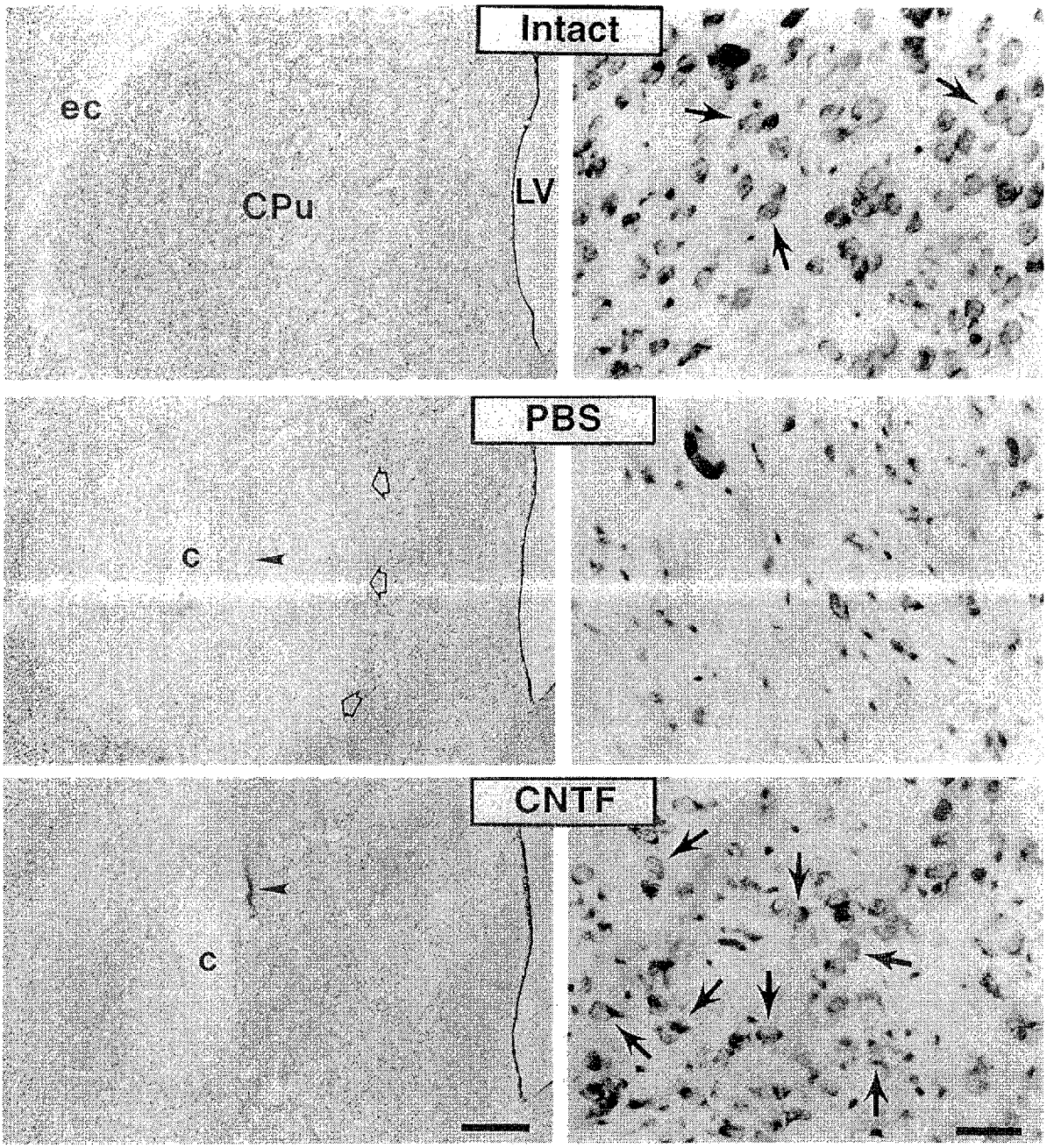

FIG. 2. Representative Nissl-stained sections (coronal plane) from brains treated with CNTF or PBS and injected with quinolinic acid. (*Upper left*) View of an untreated, intact caudate putamen (CPu). (*Upper right*) Higher magnification view of the lateral CPu showing numerous medium-sized neurons, a few of which are indicated by arrows. (*Middle* and *lower left*) The left CPu in brains treated with PBS or CNTF and injected with quinolinic acid. The two tracks in the CPu were left by the PBS or CNTF infusion cannula (c) and the quinolinic acid injection needle (arrowhead); open arrows indicate the medial boundary of the lesion. (*Middle* and *lower right*) Higher magnification views 250 μm lateral to the cannula illustrating the virtually complete absence of medium-sized striatal neurons in the PBS-treated brain (neuron loss score = 4) and the presence of numerous, normal-appearing neurons in the CNTF-treated brain (some of the surviving neurons are indicated by arrows; neuron loss score = 2). ec, external capsule; LV, lateral ventricle. (Left scale bar = 0.5 mm; right scale bar = 30 μm.)

or as assessed by assignment of semi-quantitative neuron loss scores (Fig. 3). Surviving neurons in CNTF-treated brains were disseminated throughout the striatal area affected by the quinolinic acid injection.

Given the favorable effect demonstrated by CNTF, a similar experiment was conducted using a polypeptide CNTF receptor agonist, Ax1 (24). As observed after administration of CNTF, infusion of Ax1 (4.8 μg/day) resulted in significant sparing of medium-sized striatal neurons exposed to quinolinic acid (Fig. 3). This result supports the conclusion that CNTF receptor-mediated mechanisms effect protection of striatal neurons from NMDA receptor-mediated excitotoxicity.

The neuroprotective effect of CNTF or Ax1 was achieved without apparent adverse effects on behavior or health, as indicated, for example, by body weight. Body weights measured at the end of the experiments were not significantly affected by CNTF or Ax1 treatment (unpaired *t* test). The mean body weights (±SEM) of the trophic factor-treated and the vehicle-

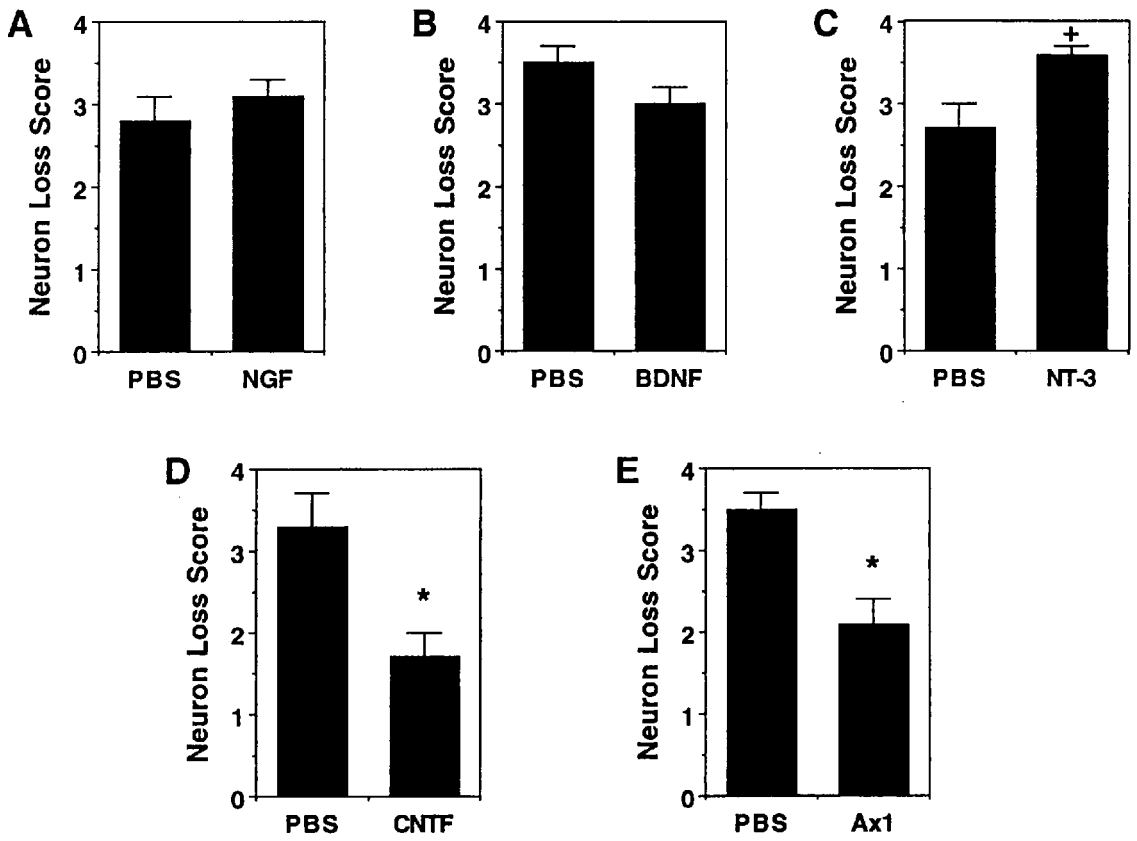

FIG. 3. Effect of treatment with neurotrophic factors on medium-sized striatal neuron loss induced by intrastriatal injection of quinolinic acid (QA). Mean neuron loss scores (±SEM) for groups treated with neurotrophic factor or PBS and injected with quinolinic acid. The number of rats in each trophic factor-treated group is as follows: NGF = 5 (*A*); BDNF = 12 (*B*); NT-3 = 10 (*C*); CNTF = 3 (*D*); Ax1 = 7 (*E*); equivalent numbers were used in the PBS-treated control groups in each experiment. Statistical comparisons were by unpaired *t* test. NT-3 treatment resulted in a significantly greater (+) mean neuron loss score compared with the PBS-treated group: $t(17) = 2.75$, $P = 0.01$. CNTF or Ax1 treatment resulted in significantly lower (*) mean neuron loss scores compared with PBS-treated groups: $t(5) = 2.7$, $P = 0.04$ and $t(13) = 4.2$, $P = 0.001$, respectively.

treated groups in the CNTF experiment were 369 ± 20 g and 331 ± 15 g, respectively ($P = 0.21$); mean body weights in the Ax1 experiment were 431 ± 26 g and 453 ± 14 g, respectively ($P = 0.44$).

Two additional experiments were performed to determine whether the neuroprotective effect of CNTF receptor ligands might persist after termination of neurotrophic factor administration, and whether treatment is effective when a lower dose of trophic factor is delivered intermittently. In the first experiment, rats were infused intrastriatally with Ax1 (4.8 μg/day) or vehicle for 3 days and then delivery was terminated by removal of the osmotic pump. Quinolinic acid was injected into the striatum 3 days thereafter (Fig. 4*A*). In the second experiment, rats received a daily intrastriatal injection of Ax1 (0.4 μg/day) or vehicle for 3 days before and 1 day after intrastriatal injection of quinolinic acid (Fig. 4*B*); thus, these rats received a total of only 1.6 μg of Ax1. In both experiments, microscope analysis of Nissl-stained sections showed significant sparing of medium-sized striatal neurons in Ax1-treated brains that was comparable to sparing seen when CNTF or Ax1 were infused continuously for the duration of the experiment (Fig. 4).

DISCUSSION

Because over 90% of the neurons in the striatum are medium-sized, GABAergic, striatonigral, and striatopallidal projection neurons (28), the present results show that treatment with CNTF or a CNTF receptor agonist protects striatal output neurons against excitotoxic insult. Thus, CNTF is one of the first purified trophic factors demonstrated to protect striatal output neurons after pharmacological application in an adult animal model of Huntington disease. Among other factors characterized, only treatment with basic fibroblast growth factor has been reported to diminish the size of a striatal lesion induced by injection of NMDA or malonic acid in adult and neonatal rats (29, 30). Although NGF-secreting fibroblasts implanted near the striatum have been shown to protect medium-sized striatal neurons from quinolinic acid in rats (31, 32), we obtained no survival-promoting effect on these neurons with purified NGF, in agreement with several earlier studies (5, 10, 33). This finding suggests that NGF is not the sole mediator of the neuroprotection provided by NGF-secreting fibroblasts. We did, however, observe that the large, darkly staining, presumably cholinergic interneurons were more prominent in NGF-treated brains, as previously reported (5, 33, 34).

Striatal expression of the high-affinity NGF receptor, TrkA, is restricted to cholinergic interneurons (35), consistent with the finding of a selective action of NGF on these neurons, whereas the high-affinity receptors for BDNF and NT-3 (TrkB and TrkC) are expressed by numerous medium-sized striatal neurons (36). BDNF and NT-3 (unlike NGF) promote the survival and phenotypic differentiation of embryonic, GABAergic, striatal output neurons *in vitro* (20, 21). Moreover, these neurotrophins can protect certain neuron populations from glutamate toxicity *in vitro* (37–39). Nevertheless, infusion of BDNF or NT-3 does not appear to protect striatal

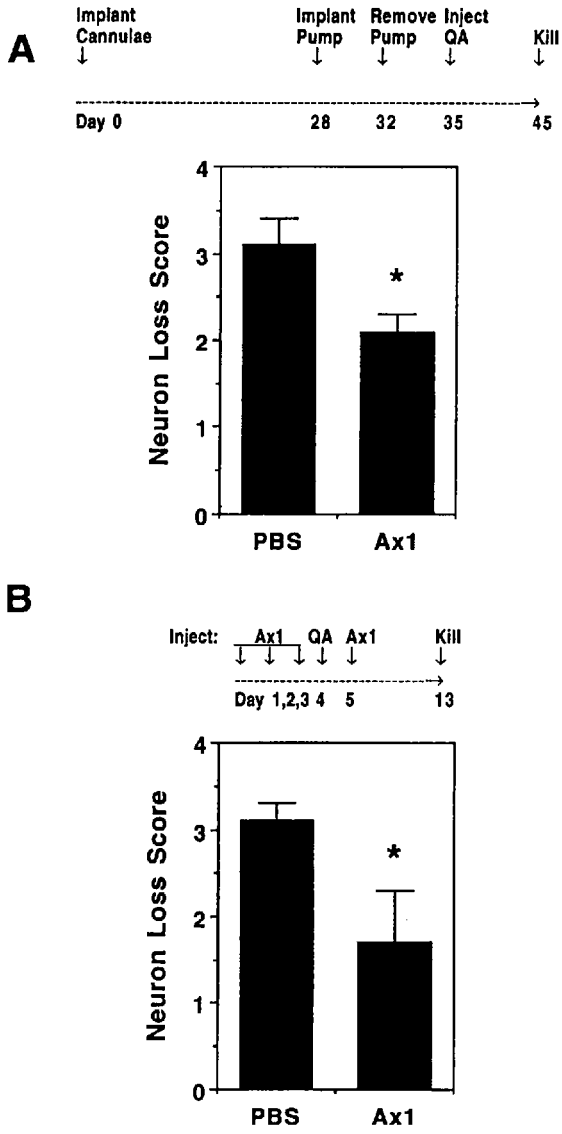

FIG. 4. Effect of treatment with Ax1 on medium-sized striatal neuron loss induced by intrastriatal injection of quinolinic acid (QA). Above each graph, a time line indicates the experimental scheme. (*A*) Mean neuron loss score (±SEM) for groups treated with Ax1 ($n = 6$) or PBS ($n = 5$) in an experimental paradigm similar to that described in the *Materials and Methods*, except the osmotic pump was implanted for only 4 days and the injection of quinolinic acid was given 3 days after removal of the pump. (*B*) Mean neuron loss score (±SEM) for groups receiving a daily intrastriatal injection of Ax1 ($n = 6$) or PBS ($n = 6$) for 3 days before and 1 day after an injection of quinolinic acid. *, Unpaired *t* test; (*A*) $t(9) = 2.5$, $P = 0.03$; (*B*) $t(10) = 2.3$, $P = 0.04$.

output neurons against NMDA receptor-mediated excitotoxicity *in vivo*, although intracerebral infusion of BDNF or NT-3 at comparable doses elicits pronounced biological effects in the striatum and elsewhere in the brain (8). The contrasting results between *in vivo* and *in vitro* studies may be explained by differences in neuron type (striatal vs. hippocampal, cortical, or cerebellar), a difference in the developmental stage of the neurons (adult vs. embryonic), or the presence of glutamatergic synaptic input *in vivo*.

The neuroprotective effect displayed by CNTF receptor ligands may occur through direct action on medium-sized striatal neurons, since there is abundant expression of mRNA for components of the CNTF receptor (CNTFRα, LIFRβ, gp130) in the striatum (40, 41). Potential mechanisms might include alteration of the expression or function of glutamate receptors, thereby modifying neuron sensitivity to glutamatergic stimulation, or enhancement of the neuron's capacity to regulate the cytosolic concentration of calcium ion, an increase in which is thought to be a critical event initiating the neurodegenerative process (42). The possibility that CNTF acts as a glutamate receptor antagonist to block quinolinic acid toxicity is unlikely, because CNTF does not block the toxic effects of glutamate *in vitro* (43).

On the other hand, CNTF receptor ligands could potentially act indirectly, by means of other components of the striatum. For example, elimination of nigral or cortical input to the striatum before exposure to quinolinic acid results in a significant reduction in the loss of striatal neurons (1, 44), indicating that the combined actions of exogenous toxin and endogenous neurotransmitters are required to induce cell death. Thus, a reduction in synaptic transmission at either glutamatergic or dopaminergic synapses would likely protect striatal neurons from an injection of quinolinic acid. Although astrocytes do not normally express detectable CNTFRα *in vivo* (40), astrocytes do express all CNTF receptor components when activated by brain injury or when maintained *in vitro* (41). Furthermore, intracerebral delivery of CNTF appears to activate astrocytes 10–48 hr after exposure, as indicated by the increased content of glial fibrillary acidic protein and its mRNA (45, 46). Whether activated indirectly or directly by CNTF, astrocytes might promote neuron survival through enhanced sequestration of excitatory amino acids or by release of substances that protect neurons.

In this study the striatal neuron populations protected from excitotoxic damage by CNTF receptor-mediated events are the same types selectively lost in Huntington disease (3). This disease has recently been associated with a defect in a gene, IT15 that encodes a 348-kDa protein, huntingtin (47, 48). Neuron populations throughout the brain express this gene, as do cells in many nonneural tissues, and thus the neurodegenerative process underlying the preferential loss of striatal output neurons remains unclear (49–51). However, a potential link between excitotoxic stimulation and increased expression of the Huntington disease gene has recently been suggested (52, 53). Although extensive studies are in progress to identify the mechanisms by which this genetic defect leads to Huntington disease, existing lines of evidence clearly implicate a role for NMDA receptor-mediated excitotoxicity (1).

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Brain-derived neurotrophic factor promotes differentiation of striatal GABAergic neurons.

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Abstract

The influence of neurotrophins on GABAergic properties of developing striatal neurons was investigated both in vivo and in vitro. Brain-derived neurotrophic factor (BDNF) specifically elevated cellular GABA content in striatal culture without altering neuronal survival. Neurotrophin-5 produced a similar effect on GABA, but nerve growth factor and neurotrophin-3 had no effect. An increase in GABA content in the striatum was also observed following BDNF injections into the cerebroventricle of neonatal rats. The increase of GABA levels in culture mainly resulted from an increase in holoenzyme activity of the GABA synthetic enzyme glutamic acid decarboxylase (GAD) and elevation of GABA uptake activity. In BDNF-treated striatal cultures, the newly differentiated neurons extended elaborate neurites and exhibited strong GAD immunoreactivity. These alterations were presumably caused by the upregulation of mRNA encoding GAD67 and the neuronal GABA transporter GAT-1. BDNF treatment also promoted other phenotypic differentiation of striatal neurons; BDNF increased the frequency of parvalbumin-immunoreactive neurons and calbindin-immunoreactive neurons and neuropeptide content for neuropeptide Y and somatostatin. These observations suggest that neurotrophins may contribute to phenotypic differentiation of GABAergic neurons in the developing striatum.

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The neurotrophins BDNF, NT-3 and NT-4/5 promote survival and morphological and biochemical differentiation of striatal neurons in vitro.

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Abstract

The neurotrophins, brain-derived neurotrophic factor (BDNF), neurotrophin-3 (NT-3), neurotrophin 4/5 (NT-4/5) and nerve growth factor (NGF), were compared for their effects on the survival and differentiation of embryonic rat striatal neurons grown in low-density cultures. Treatment with BDNF for 8 days resulted in a 40% increase in overall neuronal survival, a 3- to 5-fold increase in the number of calbindin-immunoreactive neurons, and an 80% increase in GABA-positive neurons. Treatment with NT-3 or NT-4/5 produced a 2- to 3-fold increase in the number of calbindin-positive neurons and an increase in GABA-positive cell number similar to that induced by BDNF, BDNF treatment produced a striking morphological differentiation of striatal GABAergic neurons, which was characterized by a doubling of the number of neurite branch points, the total area of aborization and the perikaryal area compared to control cultures. All three of these factors increased high-affinity GABA uptake 2-fold. NGF had no effect on any of the parameters examined. Our results show that BDNF, NT-3 and NT-4/5 promote the survival and/or differentiation of calbindin-immunopositive and GABAergic striatal neurons.

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Lentiviral delivery of Meteorin protects striatal neurons against excitotoxicity and reverses motor deficits in the quinolinic acid rat model

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ABSTRACT

Meteorin is a newly discovered secreted protein involved in both glial and neuronal cell differentiation, as well as in cerebral angiogenesis during development; but effects in the adult nervous system are unknown. The growth factor-like properties and expression of Meteorin during the development of the nervous system raises the possibility that it might possess important neuroprotective or regenerative capabilities. This report is the first demonstration that Meteorin has potent neuroprotective effects *in vivo*. Lentiviral-mediated striatal delivery of Meteorin to rats two weeks prior to injections of quinolinic acid (QA) dramatically reduced the loss of striatal neurons. The cellular protection afforded by Meteorin was associated with normalization of neurological performance on spontaneous forelimb placing and cylinder behavioral tests and a complete protection against QA-induced weight loss. These benefits were comparable in magnitude to those obtained with lentiviral-mediated delivery of ciliary neurotrophic factor (CNTF), a protein with known neuroprotective properties in the same model system. In naive animals, endogenous levels of both Meteorin and CNTF were increased in glial cells in response to QA lesion indicating that Meteorin may exert its protective effects as part of the reactive gliosis cascade in the injured brain. In summary, these data demonstrate that Meteorin strongly protects striatal neurons and deserves additional evaluation as a novel therapeutic for the treatment of neurological disorders with an excitotoxic component such as Huntington's Disease.

Introduction

Neurotrophic factors are responsible for growth and survival of neurons during development, and for maintaining adult neurons. These secreted factors are also capable of repairing specific damaged neuronal populations making them attractive drug candidates for the treatment of neurodegenerative disorders (Levy et al., 2005). As an example, Glial Cell Line-derived Neurotrophic Factor (GDNF) and Neurturin have neuroprotective as well as neurorestorative effects on dopaminergic neurons and are therefore considered promising therapeutics in relation to Parkinson's disease (PD). Recently, both members of a novel growth factor family, Mesencephalic astrocyte-derived neurotrophic factor (MANF) and cerebral dopamine neurotrophic factor (CDNF), have demonstrated equally potent effects in animal models of PD (Lindholm et al., 2007; Voutilainen et al., 2009). This illustrates the importance of finding and testing novel neurotrophic factors in relevant *in vivo* disease models.

Meteorin is a newly identified neurotrophic factor that deserves attention. During mouse development, Meteorin is widely expressed in the nervous system including the ganglionic eminence, which gives rise to a number of basal ganglia nuclei including the striatal projection neurons that degenerate in Huntington's Disease (HD) (Nishino et al., 2004). In the adult mouse brain, low levels of Meteorin are expressed by ubiquitously distributed astrocytes while higher levels are found in Bergmann glia and a few discrete neuronal populations (Jorgensen et al., 2009). Meteorin is involved in both glial and neuronal cell differentiation and has a role in cerebral angiogenesis and vascular maturation (Nishino et al., 2004; Park et al., 2008). The receptor for Meteorin is unknown but the gp130 co-receptor is involved as an upstream transducer of Jak-STAT3 signalling (Lee et al., 2010), indicating that Meteorin shares at least a part of the intracellular signalling machinery with CNTF. The therapeutic potential and *in vivo* function within the nervous system of Meteorin is hitherto unknown. With the idea that neurotrophic factors developmentally expressed in regions of the central nervous system may

Abbreviations: CDNF, cerebral dopamine neurotrophic factor; CNTF, ciliary neurotrophic factor; GDNF, Glial Cell Line-derived Neurotrophic Factor; GFP, green fluorescent protein; HD, Huntington's Disease; MANF, mesencephalic astrocyte-derived neurotrophic factor; PD, Parkinson's disease; QA, quinolinic acid.

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confer neuroprotective and restorative effects when reapplied in the same regions during disease states, we hypothesized that Meteorin may exert beneficial effects on striatal neurons.

Excitoxicity has been suggested to play a role in the neurodegeneration associated with HD (Estrada Sanchez et al., 2008). Intrastriatal injections of exitotoxins such as quinolinic acid (QA) produce a pattern of cell death that mimics the selective neuronal vulnerability seen in HD patients with loss of striatal neurons (Beal et al., 1986). Therefore, the QA model has frequently been used in both rodents and primates to examine the ability of neurotrophic factors to prevent or minimize the loss of striatal neurons and the associated motor and cognitive deficits. In particular CNTF (de Almeida et al., 2001; Emerich et al., 1996, 1997, 1998; Mittoux et al., 2000) and also BDNF (Zuccato and Cattaneo, 2009) as well as GDNF (Kells et al., 2004; McBride et al., 2003, 2006) and Neurturin (Perez-Navarro et al., 2000; Ramaswamy et al., 2007, 2009) have received much attention. Despite identifying several neurotrophic factors with the potential to exert neuroprotective effects, there is an ongoing need to identify molecules with therapeutic potential.

In the present study we first examined the expression of Meteorin in QA lesioned rats and found elevated levels in glial cells in a pattern similar to CNTF. Next, we used lenti-viral mediated striatal delivery to evaluate the therapeutic effects of Meteorin on the anatomical and functional consequences of QA lesions. The results show that over-expression of Meteorin attenuates both neurodegeneration and behavioral impairments to the same extent as CNTF. As such, Meteorin deserves additional evaluation as a therapeutic for HD and potentially other neurological disorders where excitoxicity is involved.

Materials and methods

Cloning and lentivirus production

The coding sequence of Meteorin (NM_133719) from mouse was PCR amplified and cloned *BamHI/XhoI* in pHsCXW (Leander et al., 2005) with an optimized 5′ Kozak sequence. Genomic human CNTF was PCR amplified, fused to murine immunoglobulin heavy-chain signal peptide (Emerich et al., 1996) and cloned in pHsCXW (*BamHI/XhoI*). The pHsCXW transfer vector carrying green fluorescent protein (GFP) was as used before (Fjord-Larsen et al., 2005). All constructs were verified by DNA sequencing (MWG Biotech AG, Germany) and lentivirus was produced as previously described (Leander et al., 2005).

In vitro transduction and western blotting

HEK293 cells incubated at 37 °C with a humidified atmosphere containing 5% CO_2 were transduced at MOI 1.5 and conditioned media collected after two days. Equal amounts of protein samples were loaded on 15% homogenous SDS-PAGE gels, electrophoresed and electroblotted to the PVDF membranes for western analysis. Primary antibodies for detection were Anti-human CNTF (R&D Systems, AF-257-NA, 1:500) and Anti-mouse Meteorin (R&D Systems, AF3475, 1:400). HRP-linked anti-goat (Dako, P0449, 1:2000) was used as a secondary antibody and blots developed with ECL detection system from Amersham.

Animals

All experimental procedures were reviewed and approved by the Institutional Animal Care and Use Committee and the study was conducted in compliance with the American Association for Accreditation of Laboratory Animal Care. Male Sprague–Dawley rats (N = 40; Harlan) 2–3 months old and weighing 250–300 g were housed in pairs in polypropylene cages with free access to food and water. The vivarium was maintained on a 12 hour light:12 hour dark cycle with a room temperature of 22 ± 1 °C and relative humidity level of 50 ± 5%.

Surgery

Prior to surgery, rats were anesthetized with isoflurane (3–4%) and positioned in a stereotaxic instrument (Kopf, Tujunga CA). A midline incision was made in the scalp and a hole drilled through the skull for injection of QA into the left striatum. 225 nmol of QA (Sigma) was injected using a 28-gauge Hamilton syringe mounted to the stereotaxic frame at the following coordinates with respect to bregma: (1) AP = +0.5 mm, ML = 3.4 mm, and DV = −5.5 mm. QA was infused in a total volume of 1 μL for over 5 min. The injection cannula was left in place for an additional 2 min to allow the QA to diffuse from the needle tip, after which the cannula was removed, and the skin sutured closed. Animals were sacrificed four weeks post QA injection.

In a second round of experiments, lenti virus expressing GFP, CNTF or Meteorin was injected prior to the QA lesion. Four virus deposits (0.75 μL/deposit, 2.4×10^5 transforming units total) were made along two needle tracts at the following coordinates with respect to bregma: (1) AP = +1.0 mm, ML = +2.6 mm; and (2) AP = +0.0 mm, ML = +3.7 mm, and DV1 = −5.0 mm, DV2 = −4.5. Following injection, the skin was sutured closed. 14 days following virus injection, all animals were anesthetized, placed into the stereotaxic instrument and unilaterally injected with 225 mmol QA as described before. These treatments resulted in the formation of three experimental groups: GFP (N = 10), CNTF (N = 10) and Meteorin (N = 10). CNTF function as a positive control since this is the best studied and most potent neurotrophic factor in the QA model (de Almeida et al., 2001; Emerich et al., 1998). No neuroprotective effect is expected of GFP, which accordingly function as a negative control.

Behavioral testing

Striatal dysfunction was assessed using the cylinder and forelimb placing tests (Schallert and Woodlee, 2005). Animals were tested immediately prior to QA lesion as well as at 2 and 4 weeks following lesions. The performance of the contralateral (impaired) limb was compared against the ipsilateral (intact) limb. All behavioral analysis was performed in a blinded fashion.

Forelimb placing

Briefly, the subject was held parallel to the edge of a table with the limbs hanging unsupported. As the vibrissae are brushed against the table edge, a naive rat will typically respond by placing the forelimb on top of the table. Each rat received 10 consecutive trials with each forelimb and the total number of times the rat successfully placed its forelimb onto the tabletop was recorded. A score of 10 means that the animal responded correctly on every trial, indicating normal neurologic function.

Cylinder test

In a dark testing room, rats were placed individually in a transparent cylinder (20 cm in diameter and 45 cm in height). Normal rodent behavior is to rear up on their hind limbs and explore the inner cylinder surface with their front paws. Left and right forepaw contacts with the wall of the cylinder were quantified for a total of 20 contacts during each test session with each animal. Typically, naive rats will use both front paws equally, translating to a score of 50%.

Sacrifice and perfusion

Animals were euthanized by CO_2 overdose and immediately perfused with 250 mL cold, sterile-filtered isotonic saline through the left ventricle, followed by 250 mL sterile-filtered Zamboni's fixative. The brains were removed and post-fixed in Zamboni's fixative for 48 h before cryoprotection in a 30% (W/V) sucrose solution in 0.1 M sodium phosphate-buffered saline.

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Immunohistochemistry

The immunohistochemical analysis was performed using brain tissue from the rats described before. Briefly, frozen brains were cut in six series of 40 μm coronal sections on a sliding microtome. After quenching of endogenous peroxidase activity through incubation with 3% H_2O_2, free-floating sections were incubated overnight at room temperature with primary antibody diluted in 0.1 M potassium phosphate buffered saline (KPBS) with 0.25% Triton X-100 and 2 or 5% serum from the species the secondary antibody was raised in. Primary antibodies and dilutions used in this study were as follows; Anti-GFP (Chemicon, #AB 16901, 1:5000), Anti-human CNTF (R&D Systems, AF-257-NA, 1:500), Anti-mouse Meteorin (R&D Systems, AF3475, 1:500) and Anti-NeuN (Chemicon, MAB377, 1:4000), Anti-s100β (Sigma; 1:100). Omission of primary antibody was included as a negative control. For chromogenic detection of single antibodies, sections were incubated for 2 h with biotinylated horse-anti-goat (H + L) (Vector, BA9500) or biotinylated horse-anti-mouse (Vector, BA2001) as appropriate in KPBS (0.25% triton X-100) followed by conjugation of horseradish peroxidase (HRP) using a streptavidin-HRP complex (ABC elite kit, Vector Laboratories). Finally, incubation with di-amino-benzidine (DAB), and precipitation of the chromophore with 3% H_2O_2 was used for visualization. For simultaneous analysis of multiple antibodies, sections were incubated for 2 h with Dylight488-anti-goat, Dylight549-anti-rabbit and Dylight633-anti-mouse (Jackson Immunoresearch; 1:200; all produced in donkey). Labeled sections were slide mounted and cover-slipped for microscopic analysis. Images were taken using an Olympus BX61 microscope for DAB-labeled material or a laser-scanning confocal microscope for visualization of multiple fluorescent labels. Whole brain images were prepared using a Hamamatsu Nanozoomer 1.0-HT scanner.

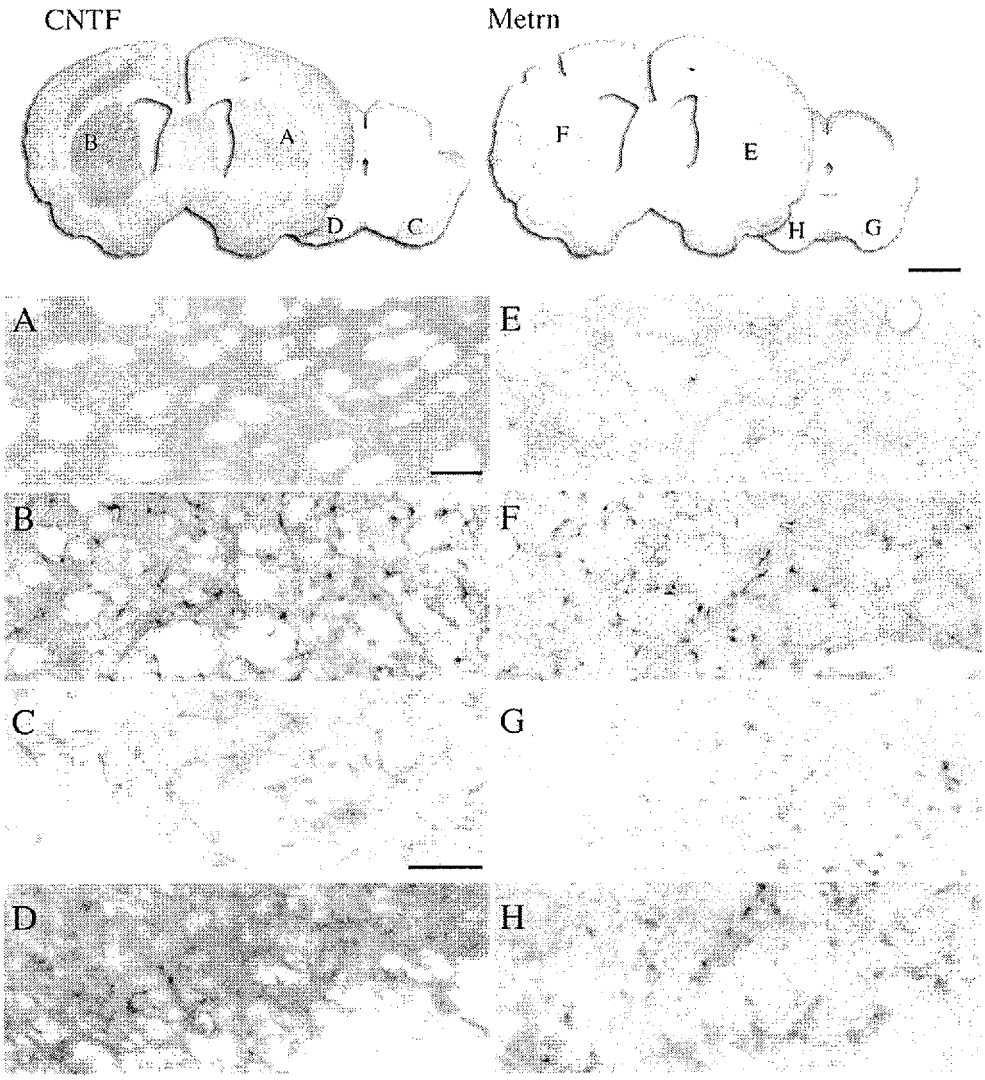

Fig. 1. Glial up-regulation of CNTF and Meteorin in response to quinolinic acid lesion. Macroscopic overviews of immunohistochemistry for CNTF and Meteorin in coronal sections at the level of the striatum and midbrain illustrate up-regulation of these proteins on the lesioned (left) relative to intact (right) hemisphere. The labeled areas (A–H) are shown at higher magnification and illustrate CNTF (A–D) and Meteorin (E–H). At the level of the striatum there are no detectable CNTF-expressing cells (A) and a small number of Meteorin-expressing cells (E). In the lesioned striatum there is a prominent up-regulation of both CNTF (B) and Meteorin (E) in cells with a glial morphology, including small nucleus and multiple proximal neuritic processes. The macroscopic image shows that up-regulation of CNTF was also detectable within the overlying cortex. At the level of the midbrain both CNTF (C, D) and Meteorin (G, H) were up-regulated in the lesioned (D, H) relative to intact (C, G) sides, although less prominently than at the level of the striatum. Scale bars: 2 mm for coronal sections; 100 μm A, E, B, F and C, G, D, H.

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Morphological analysis

Visiomorph (Visiopharm, Denmark) software was used for quantification of the ventricle area and lesion area on NeuN stained sections. This was done at +1.5, +0.5 (lesion site), −0.5, and −1.5 from bregma and thereby covers the striatum. Briefly, on all images from the respective experimental groups, the lesioned area and the size of the ventricle were quantified. The average area for each analysis was calculated as pixels including the standard error of mean (SEM). The researcher performing the morphological analysis was blinded to the identity of the experimental groups.

Statistics

Prism software (GraphPad, USA) was used to analyze behavioral data. Sample sets of 10 animals were compared by time and treatment using a fixed effect two-way ANOVA for each separate behavioral test within each study. Groups were compared against GFP as a control using a Bonferroni post-test, with p-values less than 0.05 indicating statistical significance. SigmaPlot (Systat, USA) was used to analyze the data on lesion and ventricle size. For each data set, groups were compared using one-way ANOVA followed by all Pairwise Multiple Comparison Procedures (Student–Newman–Keuls Method) with p-values less than 0.05 indicating statistical significance.

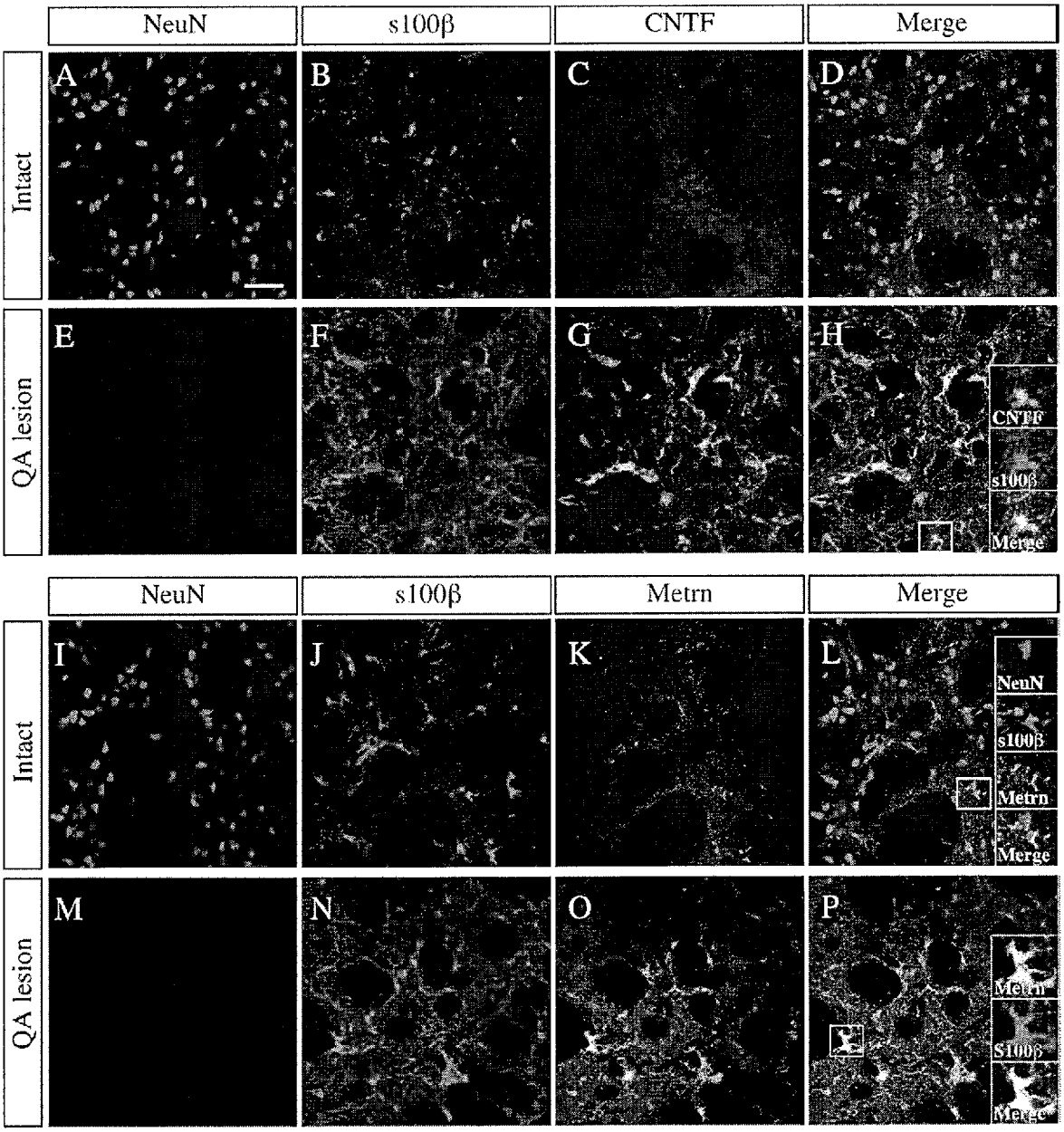

Fig. 2. Glial identity of CNTF and Meteorin expressing cells following quinolinic acid lesion. Immunohistochemistry for NeuN (A, E, I, and M; blue) and s100β (B, F, J, and N; red) along with either CNTF (C and G; green) or Meteorin (K and O; green) in the intact and lesioned striatum illustrates the robust loss of NeuN-expressing cells and concomitant up-regulation of s100β and both Meteorin and CNTF in response to the quinolinic acid injection. The merged images show that the majority of the CNTF or Meteorin expressing cells co-label with s100β. The boxed areas enlarged as insets show in finer detail the overlap between CNTF and s100β in the lesioned striatum (H); one of few examples of Meteorin and s100β in the intact striatum (L) and Meteorin and s100β in the lesioned striatum (P). Note that CNTF-expressing cells were not detected by immunohistochemistry in the intact striatum. Scale bar: 50 μm.

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Results

Meteorin expression is upregulated in glial cells in response to excitotoxic injury

Using immunohistochemistry, the endogenous expression of Meteorin and CNTF was investigated four weeks after striatal injection of QA. A robust up-regulation of both proteins was observed in the lesion side (Fig. 1). CNTF has an important role as an inducer of gliosis (Levison et al., 1996; Winter et al., 1995) and is known to be upregulated after QA injury (Haas et al., 2004). The pattern of immunoreactivity was similar for both proteins and included cells with glial morphology distributed throughout the lesioned striatum including overlying cortex, as well as throughout the corresponding pathway and terminal fields for striatal projection neurons, along the medial forebrain bundle and within the globus pallidus and substantia nigra pars reticulata. The glial identity of cells with up-regulated Meteorin or CNTF expression was confirmed by double-labelling with the glial-specific protein s100β (Fig. 2). This indicates that Meteorin is part of the glial response to excitotoxic injury and it may therefore have a neuroprotective role similar to CNTF.

Analysis of intact animals or the contralateral striatum of lesioned animals showed that any endogenous levels of Meteorin (or CNTF) in the striatum was barely detectable by the immunohistological procedures used in this study. This finding is in contrast to the expression of Meteorin in the intact mouse brain, where a low level expression ubiquitously in glia throughout the brain, including the striatum can be detected (Jorgensen et al., 2009). Interestingly, we could not detect any glial expression in the rat brain, even in the Cerebellar Bergmann glia, which are strongly Meteorin-expressing in the mouse. Outside the striatum, the neuronal expression throughout the rat brain matched well with our previous analysis in the mouse and included Meteorin-positive cells in the superior colliculus, lateral habenula, ocular motor nucleus, hypothalamus, pons and various thalamic nuclei (Fig. S1).

Effective transgene expression of Meteorin after lentiviral delivery

In order to be able to study the effects of Meteorin in the QA model, the gene was cloned and lentivirus prepared. CNTF virus was prepared as a positive control for the QA model but since CNTF itself is not a secreted protein, the genomic sequence was fused to murine

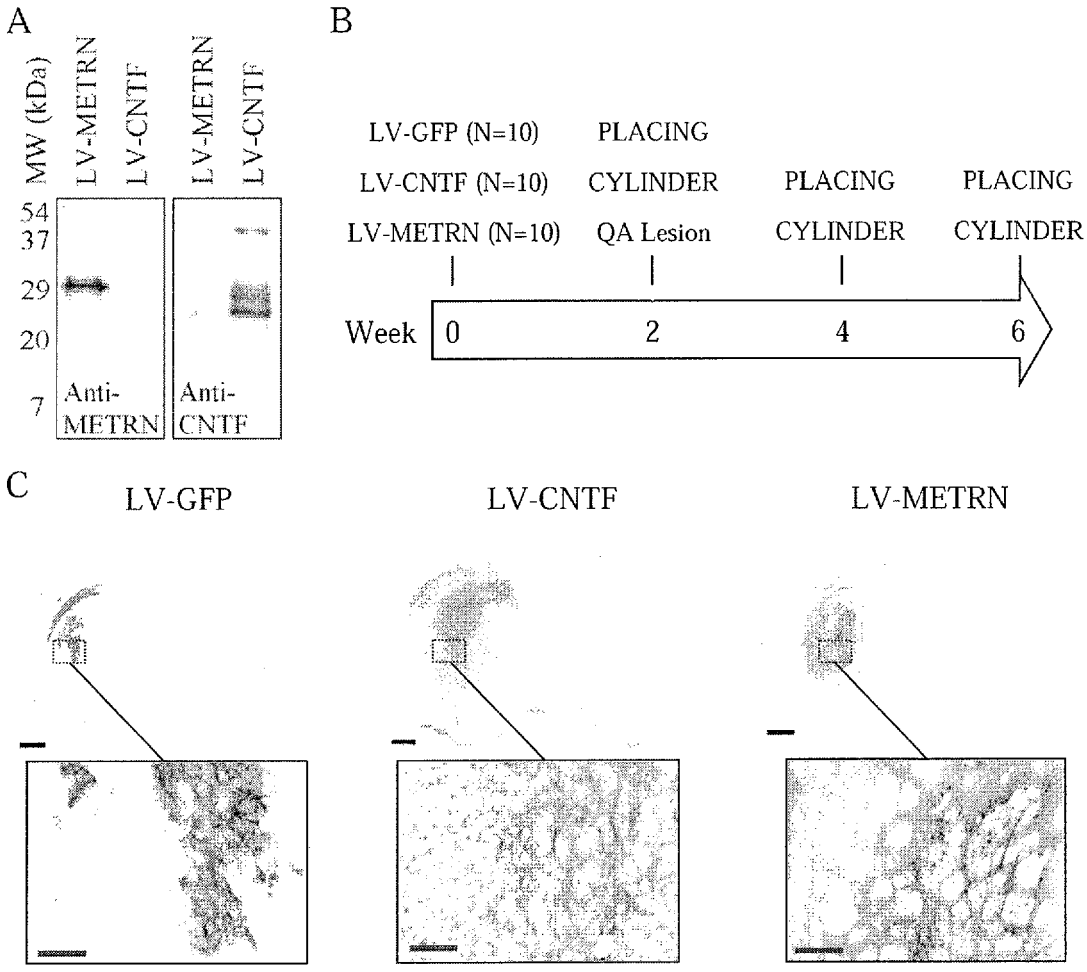

Fig. 3. A) Western blot analysis of conditioned media from HEK293 cells transduced with LV-METRN and LV-CNTF respectively. Blots were incubated with Anti-Meteorin and Anti-CNTF as indicated at the bottom. B) Timeline of experimentation. At t = 0, lenti vira expressing GFP, CNTF or Meteorin was injected into the left striatum of rats. After two weeks, animals underwent baseline behavioral testing followed by injection of QA. Behavioral assessment was repeated two and four weeks after the lesion (t = 4 and 6) and in the end animals were sacrificed and brains collected for immunohistological analysis. C) Upper whole brain images show efficient unilateral expression of all transgenes. In the individual high power magnifications, transduced cells are located to the right side and in the case of CNTF and METRN it is clear that recombinant protein is effectively secreted from transduced cells to the surrounding tissue. Note that the secreted CNTF causes additional gliosis and upregulation of its own endogenous expression. This phenomenon is not evident with METRN. Scale bar is 1 mm and 200 μm on low- and high-power images respectively**.

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immunoglobulin heavy-chain signal peptide (IgSP) (Emerich et al., 1996). To validate the vectors before *in vivo* use, HEK293 cells were transduced and the conditioned media were analyzed by western blotting. From Fig. 3A it is evident that *in vitro* transduction leads to effective secretion of Meteorin and CNTF respectively. Meteorin is secreted as a 30 kDa monomer, whereas CNTF migrates around the expected 21–28 kDa (Heinrich et al., 1998) with an additional weaker band which may represent the dimer. Lentivirus expressing GFP was prepared to be used as a negative control and validated by flow cytometry (data not shown). Next, rats were transduced as outlined in Fig. 3B and described in detail in the Materials and methods section. After the last behavioral testing (6 weeks post lenti-viral injection), sections throughout the striatum were processed for immunohistological visualization of GFP, CNTF and Meteorin. As shown in Fig. 3C, lentiviral delivery into the head of the striatum resulted in efficient transduction of the surrounding cells at the site of delivery and also along the needle track to include cells in the corpus callosum and overlying cortex. The expression of GFP was confined to the transduced cells, which is in line with the normal intracellular (cytoplasmic) distribution of the protein. In contrast, CNTF and Meteorin immunoreactivity was considerably more diffuse, covering large parts of the striatum and cortex in patterns indicative of secreted proteins in agreement with *in vitro* data.

Meteorin prevents weight loss and reverses motor deficits in QA rats

Based on daily cage side observations throughout the study, no overt signs of behavioral or neurological toxicity were observed in any animals. However, immediately after QA injection, the control animals (QA + GFP) exhibited whole-body barrel rotations, piloerection and diarrhea as previously described (Emerich et al., 2010). Also, post-QA, these same control animals lost weight relative to the CNTF and Meteorin-treated animals but thereafter gained weight at a normal rate (Fig. 4A).

Paralleling the attenuation in weight loss, Meteorin and CNTF provided significant protection against the motor deficits produced by QA. As apparent in Fig. 4B, performance of the unimpaired ipsilateral forelimb was consistent with normal function, with all treatment groups making >9.5/10 correct responses. Intrastriatal injections of QA produced significant performance deficits in the placement test. GFP control animals showed a decrease of 78.4% after 2 weeks and 78.9% after 4 weeks in the number of contralateral placements taken relative to the unimpaired limb ($P<0.01$). In contrast, a marked behavioral protection was observed in animals treated with either CNTF or Meteorin ($P<0.01$). In these animals, the impaired limb exhibited only an approximately 20% deficit. There was no significant difference between performance of the CNTF and Meteorin groups.

The results of spontaneous forelimb use in the cylinder test paralleled those observed with the placing test. At 2 and 4 weeks post-QA, a dramatic decrease in forepaw use in the limb contralateral to the injected striatum was observed in the GFP control animals ($P<0.01$) (Fig. 4C). In contrast, the animals receiving CNTF or Meteorin performed markedly better ($P<0.01$) with the controls exhibiting less than 15% contralateral limb use compared to >40% in the CNTF- and Meteorin-treated animals. No significant differences were noted in performance between the CNTF and Meteorin groups.

Meteorin protects striatal neurons, reduces lesion size, and normalizes ventricular size

QA lesioning results in a gradient of cell loss from the lesion core at the injection site to the periphery as the toxin diffuses radially. To visualize this loss of cells, brains were evaluated by immunostaining against NeuN, which is expressed in the nuclei of most neuronal cell types in the CNS (Mullen et al., 1992). In GFP control animals, the QA lesion was large enough to encompass most of the striatum at the

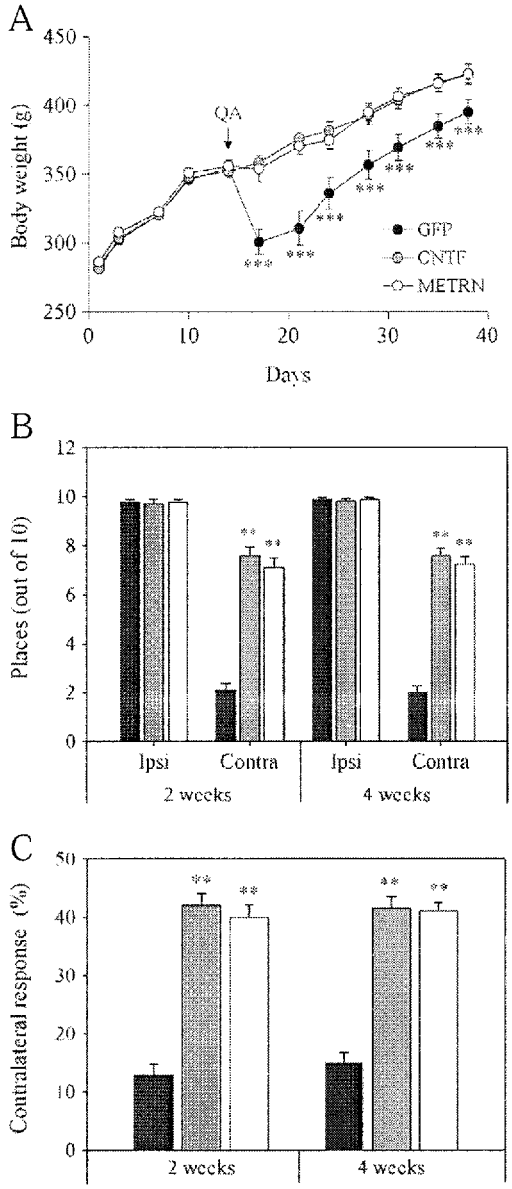

Fig. 4. Meteorin and CNTF prevent weight loss and normalize behavior in QA rats. A) Animal body weight was followed throughout the study. Note how QA injection after two weeks (↓) cause a significant (***, $P<0.001$) weight loss in the GFP group compared to CNTF and Meteorin treatment groups which seem unaffected by a steady weight increase. B) Forelimb placing test. Data are presented as mean (±SEM) number of correct places out of 10 consecutive trials with the forelimbs ipsilateral and contralateral to the striatum 2 and 4 weeks after QA (**, $P<0.01$). C) Spontaneous forelimb use in the cylinder test. Values displayed represent the percent symmetry as mean contralateral touches/total touches (±SEM) 2 and 4 weeks after QA (**, $P<0.01$). In both cases, there was no significant difference between the ipsilateral and contralateral side prior to QA injection ($t=0$, not shown). Groups are: (■) GFP; (▒) CNTF; (□) Meteorin.

level of the injection. As a consequence of the striatal cell loss, a secondary dilation of the adjacent lateral ventricle was also noted (Fig. 5). With the exception of occasional NeuN-positive debris and shrunken neurons, the lesion core was virtually devoid of NeuN-positive neurons in the GFP control group. In contrast, Meteorin significantly protected striatal neurons. In these animals, the core of the lesion was reduced and limited to a smaller, more defined area adjacent to the injection site with numerous healthy appearing NeuN-positive neurons observed immediately outside of the lesion core (Fig. 5). As shown in Fig. 6A, quantification of lesion size throughout

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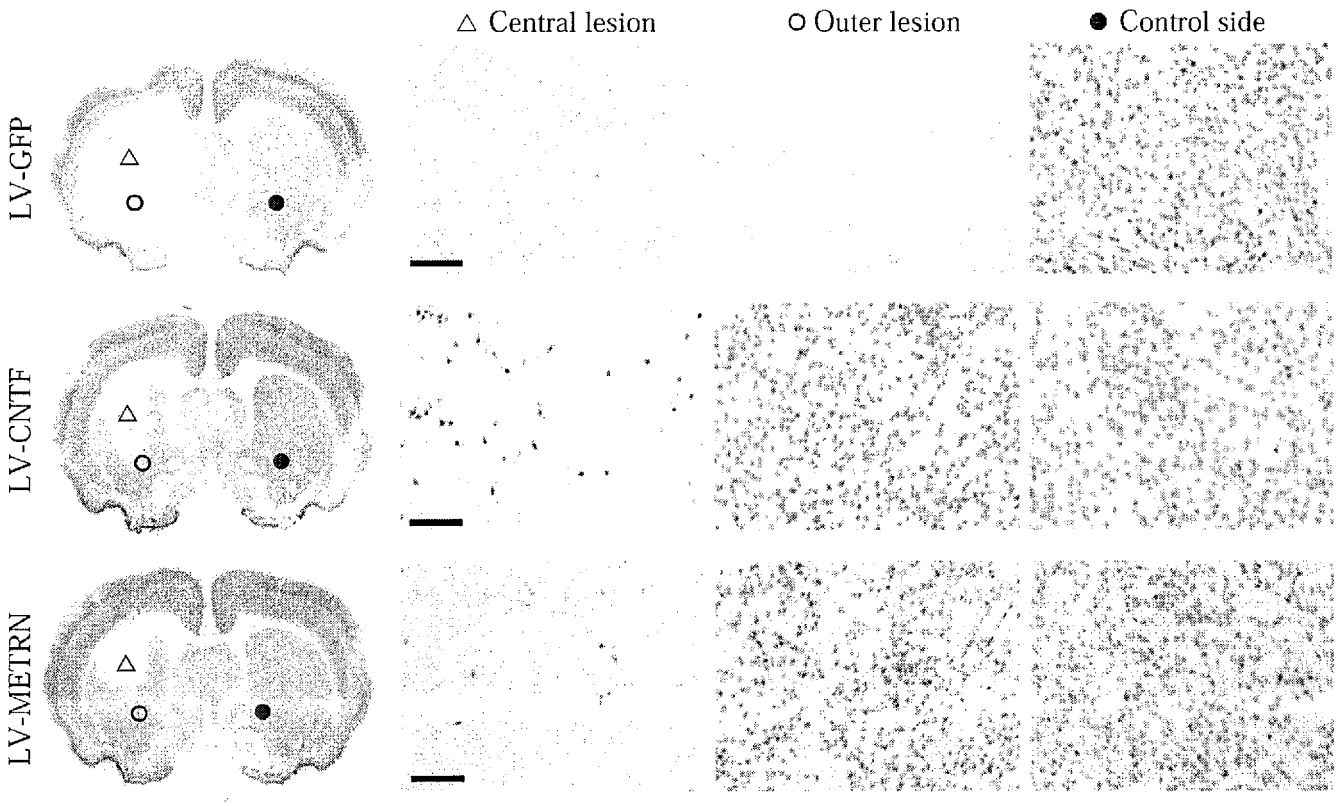

Fig. 5. Low- and high-power images of NeuN stained sections at the site of QA injection. From the whole brain sections, note the large lesion and increased size of the left ventricles in the GFP group. In the CNTF and Meteorin treated groups, the lesion is much smaller and the left ventricle appears normal. High power images are from the central lesion, outer lesion area and the corresponding control side as indicated. Scale bar is 50 μm.

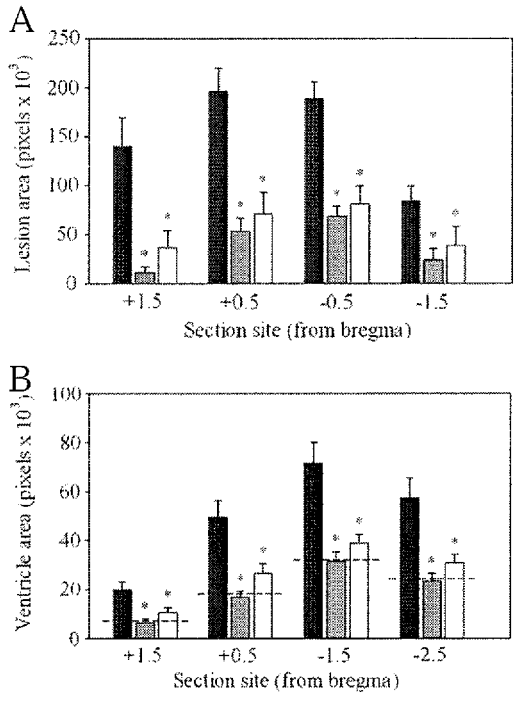

Fig. 6. A) Quantification of lesion size. QA injection was made at +0.5 mm from bregma. Sections from the lesion site as well as sections 1 mm anterior plus 1 and 2 mm posterior was analysed to cover the striatum. Note that CNTF and METRN significantly reduce lesion size at all section sites. B) Quantification of ventricular size throughout the striatum on the same sections. Average size of the ventricle in the control side is indicated by a dotted line. Note the reduction in ventricular size by CNTF and METRN. (*, $P<0.05$).

the striatum confirmed this benefit in both the CNTF and Meteorin groups. This analysis also revealed that the preservation of striatal morphology was associated with a preservation of ventricular size as the Meteorin and CNTF treated animals all had smaller and normal appearing ventricles compared to the GFP controls (Fig. 6B). Again, there was no significant difference between the Meteorin and CNTF groups.

Discussion

This study is the first to evaluate the neurotrophic potential of Meteorin *in vivo* and demonstrates that lentiviral mediated delivery to the striatum of QA lesioned rats efficiently protects striatal neurons. The size of the lesion in animals treated with Meteorin was significantly reduced compared to control animals receiving lentiviral GFP. Also, compared to the negative controls, the size of the ipsilateral ventricle was reduced in Meteorin treated animals and the size was not significantly different from the average contralateral ventricle. Injection of QA produced a marked deficit in contralateral forelimb use in the GFP group as revealed by placing and cylinder tests. In contrast, those animals receiving Meteorin showed significant improvement in both motor tests, with performance of the impaired forelimb approaching that of the intact normal forelimb. In addition, Meteorin protected against QA induced weight loss. On every parameter tested, Meteorin was equally effective as the more well-characterised neurotrophic molecule CNTF. Neuroprotection of striatal neurons by lenti-delivered CNTF has previously been demonstrated in the rat QA model (de Almeida et al., 2001) and encapsulated cell bio-delivery of CNTF has also proven effective in QA lesioned rats (Emerich et al., 1996, 1998) and monkeys (Emerich et al., 1997). Interestingly, it was recently reported that Meteorin and CNTF

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share at least part of the same intracellular signalling machinery as both molecules utilize the gp130 co-receptor as an upstream transducer of Jak-STAT3 signalling (Lee et al., 2010). Upon CNS injury, CNTF has an important role as an inducer of gliosis (Levison et al., 1996; Winter et al., 1995). In agreement with this, we observed an increase in endogenous CNTF expression by S100b positive glia in the QA lesioned striatum compared to the control side in non-treated animals. When CNTF was over-expressed by lenti-viral delivery in QA lesioned animals, we noted additional gliosis in agreement with previous studies showing that exposure to CNTF itself is sufficient to induce extensive gliosis (Levison et al., 1996). Meteorin is also up-regulated in glial cells in response to QA insult alone. However, we did not note any up-regulation of endogenous Meteorin levels or any additional gliosis by the over-expression of Meteorin. The demonstrated upregulation of Meteorin at the protein level may be due to increased mRNA stability or increased transcription. Interestingly, Meteorin transcripts are increased by CNTF treatment of retinal explant cultures (Lee et al., 2010). Therefore it is possible that the induction of CNTF in response to QA in turn upregulates the expression of Meteorin. To address this, Meteorin expression should be investigated in non-injured animals overexpressing CNTF.

Meteorin has been reported to induce glial differentiation of neural stem cells but, in contrast to CNTF, is unable by itself to induce formation of mature astrocytes (Lee et al., 2010; Nishino et al., 2004). Accordingly, Meteorin has been suggested to promote astrogenesis by acting on cells that have already committed to the astrocyte lineage rather than having an instructive role as for CNTF. Another notable difference between these molecules is that while CNTF stimulates cell proliferation this has not been the case with Meteorin in multiple *in vitro* systems (Nishino et al., 2004). Therefore, Meteorin may be able to activate glia in the adult brain without inducing cell proliferation. Activation of glia is an important hallmark as these cells release neuroprotective factors and are capable of breaking down neurotoxic metabolites thereby helping neurons to survive in the injured or diseased brain (Sofroniew and Vinters, 2010). CNTF protects against excitotoxicity by enhancing glial glutamate uptake through GLAST and GLT-1 transporters (Beurrier et al., 2010; Escartin et al., 2006). Meteorin may work in a similar fashion and it is interesting that Meteroin and GLAST expression are overlapping in the developing nervous system (Nishino et al., 2004). Previous functional *in vitro* studies have indeed suggested that the effect of Meteorin is mediated through the glia (Nishino et al., 2004;Park et al., 2008). It is also possible that Meteorin is simply released from the glia as other growth factors and cytokines in order to protect against the insult in a more direct way. In contrast to CNTF that is lacking a signal peptide, Meteorin is effectively secreted in the mature form (Jorgensen et al., 2009). In order to obtain CNTF secretion in our study we fused it to the signal peptide of the murine immunoglobulin heavy-chain. The difference in secretion is another important difference between CNTF and Meteorin which may be of biological relevance. Our study demonstrates that Meteorin is indeed a potent neurotrophic factor but further studies are needed to address the mechanism of action.

Despite the neuroprotective effects of CNTF in chemically induced HD models, no real beneficial effects has been observed in the R6/1 (Denovan-Wright et al., 2008) and YAC72 (Zala et al., 2004) genetic models of HD. Neurturin and GDNF have shown beneficial effects in 3NP (Ramaswamy et al., 2007) and QA (McBride et al., 2003) lesioned rats respectively and importantly both molecules mediate protection in transgenic N171-82Q mice (McBride et al., 2006;Ramaswamy et al., 2009). Despite identifying several neurotrophic factors with the potential to exert neuroprotective effects in HD models, many are associated with the undesired side effects and thus there is an on-going need to identify new molecules with improved therapeutic profiles. The finding that Meteorin can efficiently protect against QA induced degeneration of striatal projection neurons clearly has implications for on-going efforts to develop neuroprotective strategies for neurological conditions affecting this cell population. To further evaluate Meteorin in relation to HD, protection against mutant Huntingtin mediated toxicity should be studied *in vitro* as well as in genetic *in vivo* models where the effect on cognitive dysfunction and depressive behavior can be addressed in addition to neuroprotection. Indeed, NMDA receptor mediated excitotoxicity plays a central role in neurodegeneragtion that occurs in HD and reduction of excitotoxic stress has proven beneficial in genetic models of HD (Okamoto et al., 2009). Accordingly, it would be interesting to study the effect of Meteorin in genetic models of HD. The genetic nature of HD makes early diagnosis possible well before neurodegeneration and associated behavioral changes occur. Thus, if therapies can be devised that preserve the structural and functional integrity of the striatum, they could potentially forestall the onset and pace of functional decline in HD patients.

In addition to finding and evaluating the clinical potential for novel neurotrophic factors, it is of great importance to develop efficient delivery systems. It is anticipated that therapeutic applications of Meteorin may involve the local application of the proteins to diseased neurons in the CNS using encapsulated cell biodelivery, gene therapy, or convection enhanced delivery as this appears necessary for other growth factors such as GDNF and NGF as described in applications in PD (Lindvall and Wahlberg, 2008) and Alzheimer's disease (Lindvall and Wahlberg, 2008;Tuszynski, 2007) respectively. An important aspect in this setting is that Meteorin readily diffuses in the brain (Jorgensen et al., 2009).

In conclusion, Meteorin effectively protects against QA mediated excitotoxicity and therefore deserves additional attention as a novel therapeutic molecule for neurological disorders with excitotoxic components such as HD.

Supplementary materials related to this article can be found online at doi:10.1016/j.nbd.2010.09.003.

Conflict of interest statement

J.R.J, M.T, L.F-J, T.E.J and L.U.W. are employed by NsGene A/S.

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